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Development and utilization of transgenic New World screwworm, *Cochliomyia hominivorax*

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Abstract. The New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), was the first insect to be effectively controlled using the sterile insect technique (SIT). Recent efforts to improve SIT control of this species have centred on the development of genetically transformed strains using the *piggyBac* transposon vector system. Eight transgenic strains were produced incorporating an enhanced green fluorescent protein (EGFP) marker gene under *polyubiquitin* regulation that has the potential for use as a genetic marking system for released males. The transgenic strains were genetically and phenotypically characterized, including for life fitness parameters and mating competitiveness. These characteristics were unique for each strain and thus some strains were deemed suitable for incorporation into SIT eradication programmes. The strain with the best attributes is designated 'CLAY'. Four of the strains, including CLAY, have been successfully cryopreserved so that their original characteristics should be unchanged when further evaluation is required. With the demonstration of efficient germ-line transformation in NWS, allowing production of fit and competitive transformants, it is now possible to consider further transgenic strain development to improve SIT that are currently being tested in other dipteran species. This includes strains that allow genetic marking with fluorescent proteins, genetic sexing by female lethality, male-specific fluorescent sorting and male sterility by testis-specific lethality. The SIT may also be improved upon by new strategies resulting in lethality of offspring of released insects using conditional lethal systems based upon temperature-dependent or dietary tetracycline regulation of lethal gene expression. Both the creation of new NWS transgenic strains and the ecological safety of their release will be enhanced by new vector systems that allow specific genomic targeting of vector constructs and their subsequent immobilization, ensuring transgene and strain stability.

Key words. *Cochliomyia hominivorax*, biological control, fluorescent proteins, *piggyBac* transformation, sterile insect technique, transgenic insects.

Introduction

Transposon-mediated germ-line transformation is possible in nearly 20 non-drosophilid insect species (Handler & O'Brochta, 2005), including the New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Allen *et al.*, 2004a), which now allows the development of transgenic strains for functional genetic analysis as well as for improved and novel means of biological control. In particular, the sterile insect technique (SIT) (Knipling, 1955), which has been highly effective in eradication

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programmes, is especially amenable to improvement by transgenic strains (Robinson & Franz, 2000). These strains include those that provide fluorescent-protein (FP) marking to detect released insects, sexing for male-only strains by male-specific selectable markers or female-specific lethality, and, potentially, male sterility by testes-specific lethality. Lethality systems may be highly effective in eliminating a particular sex or tissue function, but must be highly and conditionally regulated so that strains may be reared under permissive conditions. Significant strides have been made in recent years to achieve this regulation based upon dietary components or ambient temperature conditions.

These same conditional lethality systems that may improve SIT can be extended to control insect viability, allowing the survival of populations reared under permissive conditions, but resulting in the death of offspring under non-permissive field conditions in the environment (Alphey, 2002; Handler, 2002). Such systems would not improve conventional SIT *per se*, but would, rather, provide a new means of 'sterilization'. Instead of a lack of fertility in released males, the lethality system would prevent the survival of their embryonic or larval offspring in the field. These systems, based upon temperature-dependent lethal genes or tetracycline-dependent lethal gene expression, have been described as autocidal biological control (ABC) (Fryxell & Miller, 1995) or release of insects with a dominant lethal (RIDL) (Thomas *et al.*, 2000; Alphey & Andreasen, 2002). These and similar concepts provide a new paradigm for how pest insect species may be biologically controlled, but, importantly, they depend upon the creation and release of transgenic strains which will pose biological and environmental challenges. It is the purpose of this paper to discuss the status of the transgenic strains in NWS, and the possibilities of developing new strains for improved biological control.

Materials and methods

Methods for transgenic strain development

Cochliomyia hominivorax presented unique challenges to transformation efforts. Firstly, the eggs of this insect are deposited by the female on a warm-blooded host. To collect eggs in culture, fresh ground meat warmed and maintained at body temperature must be presented to the adults. This was accomplished by placing warm ground beef on top of a container filled with warm water. The water container must be enclosed or the adults submerge and drown. The ground beef is supplemented with a small amount (200 μL) of warm aged blood. The eggs are deposited with an adhesive substance that must be removed. A method to remove the adhesive using NaOH has been described (Berkebile & Skoda, 2002).

DNA is microinjected into pre-blastoderm embryos and embryological development of *C. hominivorax* is complete after only 9 h under ideal conditions. Eggs are incubated in high humidity and high temperature (37 °C) conditions that simulate those of the warm-blooded host, resulting in rapid embryogenesis that narrows the window for injection. This effort, that resulted in the transformation of *C. hominivorax*, adjusted to rapid embryogenesis by a rapid microinjection process after thorough pre-injection preparations. Identification of alternative methods that delay early embryogenesis of *C. hominivorax*, such as limited exposure to cool temperatures, would be useful.

The larvae of *C. hominivorax* thrive as a mass in culture, whereas small numbers of larvae fail to survive. This posed a serious problem for transformation because few eggs hatch after microinjection. The successful transformation strategy used a maximum number of injected eggs per experiment to ensure that sufficient numbers of larvae would be available. Methods to ensure survival of small numbers of larvae would improve future transformation projects. Culture of the screwworm in the U.S.A. is permitted only in biological containment facilities.

Fitness parameters

Fitness parameters measured included egg production, egg hatch, average and total pupal weight, adult emergence and male: female ratio. These measurements were used to evaluate the overall fitness of each strain. Once transgenic *C. hominivorax* strains were considered to have established as stable colonies (by a minimum of five generations), fitness measurements were recorded. Transgenic strains were handled with some modified procedures. Because the verification of transgenic status is through visualization of the FP marker (PUbnlsEGFP) (Handler & Harrell, 2001b), each strain was screened under epifluorescence optics prior to pupation. The pupal and imaginal cuticles are completely opaque, making the screening of these stages impossible without destruction of the insect. For each transgenic strain, fully mature (crawler) third instar larvae were separated from rearing media and examined each generation. The procedure was usually performed on one day (day 5) during the development of each cohort of insects and unscreened pupae were discarded. Non-transgenic strains were allowed to pupate unmolested for several days; the pupae were collected on day 9 or 10. Thus, a much smaller proportion of pupae was collected for each generation of the transgenic colony, and a smaller number of transgenic adults were available to produce eggs.

Because of the handling differences between transgenic and non-transgenic *C. hominivorax* strains, certain fitness parameters were not comparable. Average pupal weight (100 pupae), egg hatch, adult emergence and male: female ratios were compared; the handling differences were assumed to have low impact on these factors. It is not known whether male and female insects pupate at different rates, but it was assumed that this was not the case.

Mating competence and competitiveness

The fluorescent marker in transgenic *C. hominivorax* strains provided a convenient mechanism for evaluating mating competitiveness in a cage containing wild-type females and equal numbers of wild-type males and homozygous transgenic males. A representative sample of eggs produced by the females was reared and the surviving larvae screened for fluorescence. Successful male mating was determined according to the

assumption that a homozygous transgenic male will father only heterozygous fluorescent offspring, whereas a wild-type male will have wild-type offspring.

Results

The first issues to resolve in the development of NWS transgenic strains for biocontrol concern whether routine and efficient germ-line transformation is possible and if the resulting transgenic insects are suitably viable and reproductively competitive. This has been well-demonstrated by the creation of eight transgenic strains using the *piggyBac* transposon vector system (Allen *et al.*, 2004a), several of which have viability parameters equal to or exceeding those of the host strain (Allen *et al.*, 2004b).

Transgenic strain development and fitness evaluation

Transgenesis technology allows recombinant DNA to introduce desired traits and characteristics into insects in order to improve their use in SIT programmes. The eight transgenic strains generated in the initial experiments were used to assess the fitness costs associated with genomic transgene integration. Because there were multiple unique integrations, and the marker protein was expected to be benign, transgenic strain quality characteristics could be compared with those of the parental strain (Allen *et al.*, 2004b; Allen & Scholl, 2005).

Some of the key issues involved in the mass release of sterile insects revolve around strain quality, including mating competence and competitiveness, of released insects. If transgenic insects are to be used in SIT, after sterilization and release they must be capable of flight, mate-seeking and mating behaviour that allows them to be reproductively competitive with wild-type individuals in the field. Within the mass rearing facility, the insects must maintain fertility and fecundity, resist infections and generally be productive. Therefore, it is critical to collect data and analyse multiple fitness traits or strain quality parameters of the transgenic insects (Table 1).

Here, we consider the phenotypes and fitness parameters of eight transgenic strains, with emphasis on four which have been cryopreserved (Leopold, 2007) and are available for further study. Strain designations were based on the first-generation transgenic parent (X, Y or Z) and the definitive area of phenotypic fluorescent expression (cuticle C, gut G, fat body F, or spiracle S). Letters were added to the two-letter designations to make pronounceable names, resulting in strains CLAY, CLIX, CLOX, COTY, FOLY, GARY, GIZA and SUEZ (Allen *et al.*, 2004a).

Cryopreserved transgenic strains

The CLAY transgenic strain had the brightest fluorescence based on visual inspection, with expression in epidermal cells beneath the cuticle spreading over the entire surface of the larva (Fig. 1). The salivary glands also expressed EGFP brightly, along with a structure at the anterior of the gut, the pharyngeal filter. The clarity of expression in this strain would make it the primary choice as a genetic marker. All strain quality characteristics measured for this strain scored better than for the parental strain except larval survival (Table 1). The only statistically significant scores were for fecundity and adult lifespan, both of which were higher than wild-type. This was the only strain tested for mating competition, and performance matched the non-transgenic parental strain. Furthermore, this strain was successfully cryopreserved and therefore may be available for use in mass production. The insertion site was amplified and sequenced (426 bases).

The CLIX and CLOX strains were derived from the same first-generation parent, but reared separately based on a perceived, but subtle, difference in EGFP expression. CLIX had a more uniform distribution of EGFP expression in the epidermal cells beneath the cuticle, like CLAY, and both strains expressed fluorescence in the salivary glands. CLOX expression beneath the cuticle was more restricted to the epidermal cells under the segmental spines. Southern blot analysis indicated that CLIX had a single transgene insert, whereas CLOX had two. Inverse polymerase chain reaction (PCR) identified a partial junction sequence from the CLIX strain, but not from CLOX. The CLIX strain was successfully cryopreserved, but the CLOX strain was lost. The CLIX strain did not have as favourable strain quality characteristics as several other strains, including CLOX (Table 1). CLIX larval survival was significantly lower than that of the control.

Table 1. Comparison of transgenic strains to parental strain (P95) of *Cochliomyia hominivorax.* Strain quality values are expressed as an absolute ratio to the control to indicate lower (negative value) or higher (positive value) quality.

Strain	Egg hatch	Larval survival	Pupal weight	Adult emergence	Fecundity	Adult lifespan	5′	3′
Control	0	0	0	0	0	0	NA	NA
CLIX	− 0.03	− 0.27*	− 0.09*	− 0.06	0.36*	0.01	No	Yes
CLOX	− 0.01	− 0.15	0.04	− 0.04	0.36*	0.04	No	No
CLAY	0.07	− 0.13	0.05	0.03	0.33*	0.18*	Yes	Yes
COTY	0.04	− 0.09	0.08*	0	0.24	− 0.04	No	Yes
FOLY	0.22*	− 0.11	0.08*	0.02	− 0.33*	− 0.17	Yes	Yes
GARY	0.03	− 0.11	0.01	− 0.06	− 0.21	− 0.09	Yes	Yes
GIZA	0.20*	− 0.21†	0.10*	0.05*	0.15	0.08	Yes	Yes
SUEZ	0.16*	0.03	0.09*	0.02	0.09	0.05	Yes	Yes

*Statistically significant.
†GIZA strain insert produces homozygous lethal.
NA, not applicable.

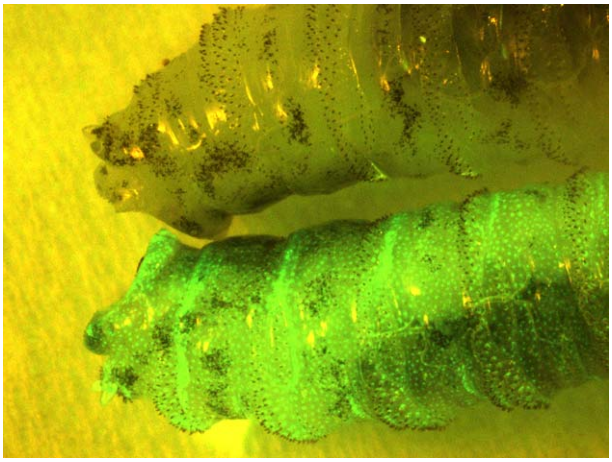

Fig. 1. Transgenic *Cochliomyia hominivorax* strain CLAY (lower) and wild-type parental strain P95 (upper) larvae under epifluorescence optics (green fluorescent protein/fluorescein isothiocyanate [GFP/FITC] filter). The posterior half of each specimen is shown. Vivid expression of green fluorescence from the PUbnlsEGFP marker gene is apparent in the transgenic strain.

The GIZA strain originated from one G1 male that produced many identical transgenic offspring. The transgenic offspring had brightly fluorescent salivary glands and a long section of fluorescent cells in the gut. No other fluorescent cells or tissues were immediately apparent, but, during screening of second-generation transgenics, it was noted that some larvae discarded as wild-type seemed to have fluorescent spots in the cells surrounding the posterior spiracles. These individuals were retrieved and used to found the SUEZ strain, and efforts were made to exclude individuals exhibiting the two combined fluorescent patterns from the GIZA strain. Thus two distinct strains were obtained. It was not possible to produce a homozygous GIZA strain, and further testing indicated that the transgene insert interrupted a vital gene, resulting in homozygous lethality. GIZA larval hatch rates were significantly higher than those of control, but the number of larvae surviving to pupation were significantly lower, and thus the homozygous lethal effect apparently occurs during larval development (Table 1). A pathology leading to larval death was never observed. Southern blots indicated a single insertion in this strain, and the insertion site was amplified and sequenced (407 bases; GenBank accession no. EU143852); the sequence translates to an uninterrupted peptide similar to a portion of *Drosophila melanogaster Myo28B* gene (expect = 2e-24, identities = 62/146 [42%], positives = 94/146 [64%], gaps = 17/146 [11%]) (Fig. 2). Clearly, this makes the strain inappropriate for release, but it was successfully cryopreserved.

This SUEZ strain, as discussed above, expressed EGFP only in the cells surrounding the spiracles. As the strain was selected, expression was more distinct and detectable around both the anterior and posterior spiracles. However, the strain was still difficult to use owing to limited fluorescence. This strain was not subjected to Southern blot analysis, but inverse PCR amplified insertion sites were sequenced. The strain quality characteristics compared favourably with those of controls, but the expression pattern precludes the use of this strain for release.

Non-cryopreserved strains

Three transgenic strains, in addition to CLOX, were not successfully cryopreserved and are therefore unavailable for further consideration and testing. We review some of their phenotypic attributes, which should be useful in the analysis and comparisons of future strains.

The FOLY strain expressed EGFP differently from the others in that it was somewhat diffuse in the posterior half of the maggot, corresponding primarily to fat body tissue and some cells of the hindgut. The quality of this strain was significantly lower than that of the parental strain in egg hatch, pupal weight and fecundity. GARY was another phenotypically distinctive strain exhibiting bright EGFP in the pharyngeal filter and a specific region of the midgut, as well as in several other tissues. Molecular analysis indicated a single transgene insert and the genomic insertion site did not appear to be a coding sequence. The quality characteristics of GARY were similar to those of the control strain, suggesting its suitability for programme use. COTY was isolated from the same mating that produced CLAY, FOLY and GARY, and may have been closely linked to FOLY. Expression of EGFP was limited to the epidermal cells under the segmental spines and was relatively faint. Salivary glands also expressed EGFP. The strain had a single transgene insert, which was partially sequenced, but had quality characteristics that compared favourably with the parental strain.

In summary, of the initial eight transgenic NWS strains created at the USDA-ARS Lincoln, Nebraska facility, only the CLAY, CLIX, GIZA and SUEZ strains were successfully cryopreserved and thus are available for further study. From initial studies, the CLAY strain is most suitable for use as a marked strain in SIT, and further evaluation and modifications of this strain will be considered.

Discussion

Transgenic strain mating competence and competitiveness

Mating competence and competitiveness will be factors critical to the success of a transgenic strain used for SIT. The fluorescent marker in transgenic *C. hominivorax* strains provided a convenient mechanism for evaluating mating competitiveness in a cage containing wild-type females and combined transgenic and wild-type males. A representative sample of eggs produced by the females was reared and the surviving larvae screened for fluorescence. A homozygous transgenic male will father only fluorescent offspring, and the wild-type male's offspring will all be wild-type. Comparisons of the transgenic strain CLAY and the P95 wild-type strain showed the CLAY transgenic males to be as competitive as P95 males.

There is concern that transgenic organisms could persist once released and generate hazards above and beyond those they were intended to control. This could occur by a failure in the

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G ATC ATA ACA ATA CAA AGA GGT TTT CGT AAA GTA TTA TTT AAA CGT TTC CTG GAT AAA
  Ile Ile Thr Ile Gln Arg Gly Phe Arg Lys Val Leu Phe Lys Arg Phe Leu Asp Lys

  TAT CGT AAA GCG GTA ATA CTA ATA CAA AAA ACT TGG AGA GGT TAT AGA GAA CGT AAA
  Tyr Arg Lys Ala Val Ile Leu Ile Gln Lys Thr Trp Arg Gly Tyr Arg Glu Arg Lys

  AAT TAT TTG GTG ATG CAT AAT GGA TTC CAT CGT TTG GCT GCC TCT GTG GCC TCA AGA
  Asn Tyr Leu Val Met His Asn Gly Phe His Arg Leu Ala Ala Ser Val Ala Ser Arg

  CAA TTA ACC TAT CGC TTT GGT TTA TTG AGA AAT CGC ATA AGT GGT TTG CAA GCT CAC
  Gln Leu Thr Tyr Arg Phe Gly Leu Leu Arg Asn Arg Ile Ser Gly Leu Gln Ala His

  TGC CGA GGT TAT TTG GTG AGA AAA GAA TTT AAA GCA AAA TAT GCT TTA AGA ATA GCA
  Cys Arg Gly Tyr Leu Val Arg Lys Glu Phe Lys Ala Phe Tyr Ala Leu Arg Ile Ala

  CGA GTG CGA GAA CTT AAA TTA TTG AGA ACA CAA GAA GAA GAA CAA TAT CGT AAG GCT
  Arg Val Arg Glu Leu Lys Leu Leu Arg Thr Gln Glu Glu Glu Gln Tyr Arg Lys Ala

  AAA GAA CGT AAT TGG AAA CAA CAT GCA GAA GAA AAT TAT CAA AAA CGT TTA AGA GGT
  Lys Glu Arg Aln Trp Lys Gln His Ala Glu Glu Aln Tyr Gln Lys Arg Leu Arg Gly

  ATT GAT C
  Ile Asp
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B

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GIZA     : 1   IITIQRGFRKVLFKRFLDKYRKAVILIQKTWRGYRERKNYLVMHNGFHRLAASVASRQLT
               I+TIQRG R+VLF+R++ +YR+A+I +Q+ WRG  +R+ Y VM  GFHRL A +A++QLT
DmMyo28B1: 745 IVTIQRGIRRVLFRRYMKRYREAIITVQRYWRGRLQRRKYQVMRQGFHRLGACIAAQQLT

GIZA     : 61  YRFGLLRNRISGLQAHCRGYLVRKEFKAKY-------------ALRIARVREL-KLLRTQ
              +F ++R R   LQA  RGYLVRK+F+ K                L++A+++E  +LLR Q
DmMyo28B1: 805 TKFTMVRCRTIKLQALSRGYLVRKDFQKKLLERRKQNQLKKEELLKLAKIKEAEELLRLQ

GIZA     : 107 EEEQYRKAKERNWKQHAEENYQKRLR 132
                 Q ++ KER  ++  E+  Q+  R
DmMyo28B1: 865 ---QLKEQKEREQREQQEKRLQEEQR 887

Score = 114 bits (285), Expect = 2e-24. Identities = 62/146 (42%), Positives
= 94/146 (64%), Gaps = 17/146 (11%).
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Fig.2. (A) Nucleotide and translated sequence (5′–3′ with respect to logical translation; transgene insert is in reverse orientation) surrounding the GIZA transgene genomic insertion site. *Sau*3AI restriction sites are in bold, as is the *piggyBac* TTAA target site. (B) BLASTn comparison between translated GIZA sequence and *Drosophila melanogaster* myosin 28B1 (gi 24582547).

transgenic insect itself, for example, by mutation or remobilization of the transgene, or by a failure in the production system, as in the inadvertent release of fertile insects. Assessment of these risks requires baseline data establishing the relative fitness of transgenic insects compared with 'wild-type', laboratory-reared counterparts. A series of experiments and analyses to compare overall fitness and mating fitness between transgenic and parental strain (P95) wild-type insects indicated that there was little or no loss of fitness in most strains of transgenic NWS. Specific strains showed some fecundity and adult emergence costs, but these were probably associated with specific insertion sites rather than the existence of the transgene itself.

Improved strains for SIT

The efficient transformation of NWS, yielding transgenic organisms with strong fitness characteristics, is supportive of creating additional transgenic strains that can be used for SIT and other means of biological control. Routine testing and creation of NWS transgenics is, however, complicated by quarantine issues and the relatively difficult rearing of this species. Thus, it is advantageous to first consider transgene constructs that have been tested in other species for the same or similar purposes. As the *piggyBac* vector and *polyubiquitin*-regulated EGFP marker system used to transform NWS was originally created for and tested in Drosophila (Handler & Harrell, 1999) and tephritid flies (Handler & Harrell, 2001b), many of the advanced vectors tested in these species should be easily transferred for use in NWS, and the development and use of these systems will be discussed.

New marked strains and use of stabilized vectors

Currently, the CLAY strain marked with EGFP has several characteristics that make it the primary candidate for release as a marked strain in SIT. However, the creation of additional, new fluorescent-marked strains is important for backup purposes, different fluorescent markers can be used to distinguish between flies from different releases, and their use in new advanced vectors can simplify strain creation and provide for greater ecological safety (Wimmer, 2005). Therefore, developing new transgenic marked strains remains a high priority. Other FP colours distinguishable from EGFP and used as transgenic markers include red (DsRed), blue (CFP) and yellow (YFP) fluorescence, and their enhanced versions (Handler & Harrell, 2001a, Horn *et al.*, 2002). Their use, and use of EGFP strains intended for release, should be within new transposon vectors that can be immobilized post-integration, which ensures the stability of the transgene vector within the host genome. This is essential to maintain strain integrity and also to minimize concerns for ecological safety (Handler, 2004). These types of vectors, which depend on post-integration remobilization of vector terminal sequences, have been created and tested in several Drosophila

and tephritid species (Handler *et al.*, 2004; Dafa'alla *et al.*, 2006). Another vector system additionally allows targeting to specific genomic insertion sites (Horn & Handler, 2005). Vector stabilization by terminus remobilization in Drosophila was achieved by mating transformants carrying the initial unstabilized vector to a jumpstarter strain with a chromosomal source of *piggyBac* transposase (Handler *et al.*, 2004). Importantly, in tephritid species where jumpstarter strains are not available, terminus remobilization was achieved efficiently by direct *piggyBac* helper plasmid injection (A. Handler & R. Krasteva, unpublished data, 2007). This had the added advantage of producing stabilized vector strains in a single generation, and this approach will be taken with NWS, for which jumpstarter strains are also unavailable.

Sperm and testes markers

A priority for SIT is to determine whether trapped female flies have mated with released males. This may be determined by having sperm marked with an FP, which can be achieved by linking the FP to a sperm-specific promoter, from genes such as *β2-tubulin* (Fackenthal *et al.*, 1993). In Drosophila, we have shown that marked sperm from transgenic β2-tubulin:DsRed males can be detected in the seminal receptacle of mated females (A. Handler & R. Harrell, unpublished, 2005). Similar detection has been shown for the Caribbean fruit fly, *Anastrepha suspensa*, where β2-tubulin:DsRed-marked sperm is detected in the female spermathecae (G. Zimowska, N. Xavier & A. Handler, unpublished, 2006). There is also the potential to use marked sperm in sperm precedence studies when multiple matings occur. The Drosophila *β2-tubulin* promoter was tested in sperm marking in the Caribbean fruit fly, but heterologous function was not apparent based on visual detection of DsRed fluorescence (G. Zimowska, N. Xavier & A. Handler, unpublished, 2006). It was assumed that, despite the relatively high conservation of *β2-tubulin* genes, promoter function was more species-specific, and thus isolation of the *β2-tubulin* gene, and proximal genomic DNA, from Caribbean fruit fly would be necessary. The gene was therefore isolated by a direct PCR approach using degenerate primers, with inverse PCR used to sequence the proximal 5′ and 3′ genomic DNA, and approximately 1.5 kb of 5′ sequence was tested for promoter activity. This was confirmed by testes-specific DsRed fluorescence observed in the sperm tails and seminal material of Caribbean fruit fly males, and in the spermathecae of non-transformed mated females. In addition, PCR was able to specifically detect marked sperm from abdominal DNA preparations of individual mated females. Existing vectors could be tested initially for use in NWS, but it is likely that isolation of the NWS *β2-tubulin* promoter will be necessary, and this should be straightforward.

Sexing for male-only strains

A high priority for programme efficiency and cost-effectiveness is the ability to sex strains early in development and, ideally, to eliminate females by the first instar larval stage. Transgenic vectors allow this to be achieved in several ways. Firstly, fluorescent body-marked vectors that integrate into the male-specific Y-chromosome will only be expressed in males. Although Y-chromosomes often represent a relatively small percentage of total genomes in dipterans, routine transformation of these species typically results in some Y integrations, and we have created three Caribbean fruit fly male-fluorescent strains (A. Handler & R. Harrell, unpublished data, 2005). Importantly, fluorescent sorters that allow high-throughput sorting of insect embryos and larvae that express specific fluorescence are available and these may be used in sexing protocols (Furlong *et al.*, 2001). Sexing based on male-specific fluorescence may also be achieved by testes-specific fluorescence using *β2-tubulin*-regulated fluorescence, which has been demonstrated in a mosquito species (Catteruccia *et al.*, 2005; Smith *et al.*, 2007). However, detection may not be feasible until the third instar larval stage, resulting in suboptimal efficiency.

A third transgenic approach towards sexing is the use of sex-specific lethality systems. These require sex-specific regulatory control systems to drive the expression of a conditional lethal system and, ideally, lethality should occur early in development. For many dipterans the ideal sex-specific regulatory system regulates sex determination gene expression, which is initiated in early embryogenesis and functions throughout development (Handler, 1992). This is based on a sex-specific transcript splicing system that has alternative male and female 3′ intron splice sites. A translational stop codon is specifically revealed in the longer male transcript, which results in a truncated, non-functional polypeptide. Use of a downstream 3′ splice site in females results in deletion of the stop codon within the intron. Several sex determination genes in Drosophila utilize this system, and cognates of the *transformer* (*tra*) and *doublesex* (*dsx*) genes are known to exist in other dipterans, and the *transformer* splicing system has been tested in several species (Saccone *et al.*, 2002). The *tra* alternative splicing cassette can be placed in-frame within a variety of conditional lethal systems, including the tet-off suppression system or several temperature-dependent lethal genes.

Sterile male strains

Currently, male sterility is achieved by pupal irradiation which, although it effectively disrupts germ-line chromosomes, also creates somatic damage that can adversely affect fitness. There are also considerable insect handling costs involved in irradiation and the production of suitable irradiators is becoming more limited. Thus, developing a genetic means of specifically sterilizing males is a high priority, which, potentially, can be efficiently achieved using recombinant constructs already described for other objectives. One of these involves having the testis-specific *β2-tubulin* promoter drive a conditional lethal system. A caveat for this approach is that production or transfer of the male seminal peptides that inhibit subsequent matings in females should not be disrupted.

Organismal lethal strains

A logical extension of conditional lethality systems that kill a specific sex or destroy a specific tissue for use in SIT is lethality

limited to insects early in development under non-permissive conditions. This could allow survival under mass rearing, with early death of the progeny of released insects after they have mated with wild insects. This can be achieved in several ways. One is by temperature-dependent lethality resulting from a dominant temperature-sensitive lethal gene or temperature-sensitive mutations of toxin subunit genes. One system based on a cold-sensitive allele of the Drosophila *Notch* gene, which would cause lethality at temperatures of ≤ 20 °C, has been described as autocidal biological control (ABC) (Fryxell & Miller, 1995). We have tested a converse system resulting in temperature-dependent lethality at high temperatures. The DTS-5 mutant allele from *Drosophila melanogaster*, which causes larval or pupal death at 29 °C (Saville & Belote, 1993), was tested by transforming it into *Ceratitis capitata*. Several *C. capitata* lines homozygous for the Drosophila DTS-5 transgene exhibited 90–95% lethality by the larval or pupal stage at 30 °C. DTS-5 is a point mutation in a highly conserved 20S proteasome subunit (Saville & Belote, 1993), and efforts to improve lethality in tephritids centre around isolating and mutagenizing the DTS-5 cognate, as well as another DTS point mutation, DTS-7 (Smyth & Belote, 1999). These conditional lethal mutations would be particularly useful in tropical and subtropical insects, and their high conservation suggests that they can be similarly isolated and mutagenized for use in NWS.

Conditional lethality may also be regulated by gene expression systems that are either turned on or turned off by a chemical supplement to the diet. Foremost among these systems is the tet-off system based on the tetracycline operon from *Escherichia coli*, where tetracycline, or an analogue, suppresses gene expression (Gossen *et al.*, 1993). A mutated tet-on version of this system acts conversely in that tetracycline is needed to promote gene expression. The tet-off system was originally tested in Drosophila as a female-specific lethality system, using the yolk protein promoter active in female fat body to ultimately drive expression of the *hid* cell death gene (Heinrich & Scott, 2000; Thomas *et al.*, 2000). Another version of this system, using embryonic promoters, resulted in both male and female lethality in Drosophila during early development in the absence of tetracycline (Horn & Wimmer, 2003). These approaches are being tested in *C. capitata* (Schetelig *et al.*, 2007) and could be extended to NWS if suitable embryonic promoters, such as cognates from the *serendipity* or *nullo* genes, were available. Potentially, the allele of the *hid* cell death gene used in Drosophila could also confer lethality in other dipterans.

Conclusions

The New World screwworm was the first insect to be successfully eradicated using the SIT. In an effort to improve future SIT programmes, research was initiated to develop transgenic insect technology. The first transformation experiments produced eight transgenic strains incorporating an EGFP marker that could be used, potentially, to identify released insects. Each stable strain had at least a single copy of the transgene incorporated into a unique genomic location and had unique genetic, phenotypic and strain quality characteristics. The original lines were produced in the ARS Biosecure facility in Lincoln, Nebraska, and the subsequent closure of this laboratory resulted in the cryopreservation of the transgenic screwworm germplasm. This was successful for four of the eight transgenic strains, one of which, CLAY, has attributes as a fluorescent marked strain that have led it to be considered for use in SIT, to unambiguously identify released insects from wild pests.

Desirable traits for further improvement of SIT for NWS include the development of male-only strains by conditional female lethality, or sexing achieved by fluorescent sorting of males with vector insertions in the male-specific Y chromosome. It may also be possible to sort males by testis-specific expression of FPs driven by promoters such as those for the *β2-tubulin* gene. These strains could also be used for sperm marking to identify females that have mated with released males and the *β2-tubulin* promoter could additionally be used with lethal genes to confer conditional male sterility.

Strain improvements for SIT using transgenesis are achievable; however, the ultimate goal for pest management using this technology is the creation of new strains that are conditionally lethal in early development. Systems have already been tested in Drosophila and tephritid species based on dominant temperature-sensitive lethality and tet-off regulation of cell death gene expression. As the transgenic conditional lethal strains were created using the same *piggyBac* vector system successfully tested in NWS, it is highly likely that similar strains can be created for NWS. This should be a high priority of future research.

Conflicts of interest

All authors declare no conflicts of interests.

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